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Cellular and Molecular Biology of Aging Endothelial Cells

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Abstract

Cardiovascular disease (CVD) is the leading cause of death in the United States and aging is a major risk factor for CVD development. One of the major age-related arterial phenotypes thought to be responsible for the development of CVD in older adults is endothelial dysfunction. Endothelial function is modulated by traditional CVD risk factors in young adults, but advancing age is independently associated with the development of vascular endothelial dysfunction. This endothelial dysfunction results from a reduction in nitric oxide bioavailability downstream of endothelial oxidative stress and inflammation that can be further modulated by traditional CVD risk factors in older adults. Greater endothelial oxidative stress with aging is a result of augmented production from the intracellular enzymes NADPH oxidase and uncoupled eNOS, as well as from mitochondrial respiration in the absence of appropriate increases in antioxidant defenses as regulated by relevant transcription factors, such as FOXO. Interestingly, it appears that NFkB, a critical inflammatory transcription factor, is sensitive to this age-related endothelial redox change and its activation induces transcription of pro-inflammatory cytokines that can further suppress endothelial function, thus creating a vicious feed-forward cycle. This review will discuss the two macro-mechanistic processes, oxidative stress and inflammation, that contribute to endothelial dysfunction with advancing age as well as the cellular and molecular events that lead to the vicious cycle of inflammation and oxidative stress in the aged endothelium. Other potential mediators of this pro-inflammatory endothelial phenotype are increases in immune or senescent cells in the vasculature. Of note, genomic instability, telomere dysfunction or DNA damage have been shown to trigger cell senescence via the p53/p21 pathway that results in increased inflammatory signaling in arteries from older adults. This review will discuss the current state of knowledge regarding the emerging concepts of senescence and genomic instability as mechanisms underlying oxidative stress and inflammation in the aged endothelium. Lastly, energy sensitive/stress resistance pathways (SIRT-1, AMPK, mTOR) are altered in endothelial cells and/or arteries with aging and these pathways may modulate endothelial function via key oxidative stress and

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inflammation-related transcription factors. This review will also discuss what is known about the role of “energy sensing” longevity pathways in modulating endothelial function with advancing age. With the growing population of older adults, elucidating the cellular and molecular mechanisms of endothelial dysfunction with age is critical to establishing appropriate and measured strategies to utilize pharmacological and lifestyle interventions aimed at alleviating CVD risk.

Keywords

Aging; Endothelium; Oxidative Stress; Inflammation; Senescence; Telomere; Genomic Instability; SIRT-1; AMPK; mTOR

1. Cardiovascular Disease, Vascular Endothelial Function, and Aging

Cardiovascular diseases (CVD), largely defined as stroke, coronary artery disease, heart failure, and cardiac arrest, are the predominant killers of Americans, accounting for ~752,000 deaths per year according to current statistics from the Centers for Disease Control [1]. CVDs cause ~35% of all deaths for Americans 65 years of age or older, making them the leading causes of death in this age group [1]. Furthermore, with advancing age, the prevalence of CVDs among Americans increases progressively, from ~5.5% in 25–44 year olds to ~41% in people 65 years of age or older [1]. Thus, CVDs can be considered true diseases of aging.

Heart disease, stroke, and hypertension are all diseases currently recognized to be caused, in part, by arterial dysfunction [2, 3]. Age-related alterations to arteries are thought to lead to a dysfunctional phenotype that precedes CVDs [2, 4, 5]. Importantly, the dysfunctional phenotype that develops in arteries with advancing age can occur in the absence of overt CVD and conventional CVD risk factors [6, 7], supporting the idea that these changes are a primary effect of aging that may be a precursor to the development of CVD. Large landmark studies, like the Baltimore Longitudinal Study on Aging (BLSA) and the Framingham Heart Study, demonstrated that the age-associated phenotype of arteries involves, among other changes, the development of a dysfunctional arterial endothelium [4, 8–10]. This dysfunctional endothelial phenotype is common to humans and non-human primates as well as rodents [11–13] and these changes contribute to a host of hemodynamic changes, including augmented large and resistance arterial tone, induction of greater oscillatory shear stress and elevated large artery stiffness[2, 10], that contribute to increases in arterial blood pressure [14] and atherosclerosis [3] seen with advancing age.

The arterial endothelium is extremely dynamic and performs many vital functions that vary from one segment of the arterial tree to another as well as from one organ system to another [15, 16]. The vascular endothelium releases molecules that act in an autocrine and paracrine manner to regulate the function and health of the vascular network. These include the maintenance of blood in a fluid state; exchange of fluid and molecules between the blood and surrounding tissues; creation of new vascular networks; participation and facilitation of the immune response; and the control of vascular resistance in response to changes in blood flow by regulating arterial tone in resistance arteries [15, 16]. A healthy vascular

endothelium is in a tightly regulated state of balance between pro- and anti-oxidants, vasodilators and vasoconstrictors, pro- and anti-inflammatory molecules, and pro- and anti-thrombotic signals. A diseased or dysfunctional endothelium that has lost its tightly regulated balance and displays pro-oxidant, vasoconstrictor, pro-inflammatory and pro-thrombotic properties. One hallmark of vascular endothelial dysfunction is impaired endothelial dependent dilation (EDD), which is predictive of future CVD events [3, 17]. Indeed, the Framingham Heart Study has recently demonstrated that *increased age is the strongest independent correlate of EDD* [18]. Therefore, it is of great clinical significance that we obtain a better understanding of the mechanisms underlying age-related decreases in endothelial function and to test the efficacy of interventions that may restore endothelial function in middle-aged and older adults.

2. Goal of the Review

The first goal of this review will be to introduce the two macro-mechanistic processes, oxidative stress and inflammation, that contribute to endothelial dysfunction in healthy older adults and rodent models. Next, we will discuss the cellular and molecular events that lead to the vicious cycle of inflammation and oxidative stress in the aged endothelium. Then, we will discuss the emerging concepts of senescence and genomic instability as it relates to the aforementioned processes. Lastly, we explore how “energy sensing” longevity pathways that appear to modulate endothelial function with advancing age. In this review, we will focus first on *in vivo* or *ex vivo* studies that directly examined endothelial cells. However, a major obstacle to our understanding of the events that lead to endothelial dysfunction with advancing age is access to pure primary endothelial cells from humans or rodent models. Next, we will consider studies utilizing whole artery homogenates with the understanding that protein expression in whole arteries is strongly biased toward the smooth muscle cell component rather than the endothelium. Lastly, this review will explore the mechanisms of endothelium dysfunction defined as reductions in EDD assessed by the response to pharmacological or physiological stimuli. Our focus on EDD is because a majority of studies that perform mechanistic studies utilize this marker. It is not to say measures of angiogenesis, permeability, fibrinolysis or other markers of endothelial function are not as important; indeed these are critical functions which are vastly understudied, but at the present time, the mechanisms leading to impairments in these functions in aged endothelial cells or their direct relation to CVD development are not clearly understood.

3. Mechanisms of Age-Associated Vascular Endothelial Dysfunction

Aging is associated with endothelial dysfunction in both men and women in the absence of clinical disease. A majority of the published studies describing this dysfunction measure EDD [19–24], but measures of fibrinolytic factors released from the endothelium [25], permeability [26] and angiogenesis [27, 28] have also been made. Multiple animal models of vascular aging phenocopy findings from human studies, have demonstrated impaired EDD [29], fibrinolysis [30], permeability [31], and angiogenesis [32] with advancing age. In older adults, several traditional fasted risk factors, i.e., elevated blood pressure [33], LDL cholesterol [34], blood glucose [35], sodium intake [36], as well as non-traditional markers, i.e., white blood cell count [37] and plasma norepinephrine [38], can modulate the severity

of endothelial dysfunction as assessed by EDD (as reviewed in depth by Seals et al.[6]). Interestingly most of these factors have been shown to modulate endothelial function via oxidative stress or inflammation, as described in detail below. Lastly, it is known that with advancing age post prandial clearance of elevated glucose and lipids decreases significantly even in healthy adults [39, 40]. Furthermore, this post prandial state is associated with acute endothelial dysfunction in young and middle aged adults [41, 42]. Thus, it is a tenable hypothesis that this post prandial state underlies the vascular aging phenotype since endothelial culture studies suggest that physiological elevations in glucose and lipids induce oxidative stress and inflammation and also negatively alter "energy sensitive" pathways [43–45]. Due to the lack of appropriate fasting time to resolve the acute metabolic insults, these alterations in apparently normal healthy middle aged and older adults may "set the table" for sustained endothelial dysfunction mediated by chronic vascular endothelial oxidative stress and inflammation. This viable hypothesis needs to be empirically tested, but would be consistent with the existing associative data.

3a. Aging, Nitric Oxide (NO) Bioavailability and Endothelial Function

In the vascular endothelium, L-arginine and the cofactors; BH4 and FADH, are necessary for the production of NO by the enzyme endothelial NO synthase (eNOS) [46]. NO is one of the most important vasodilatory and anti-atherosclerotic molecules produced by the endothelium [46–48]. The major mechanism involved in the age-related reduction in EDD is reduced vascular NO bioavailability [49]. While initially thought to be the result of reduced expression of eNOS [50–52], evidence from endothelial cells collected from human and animal arteries demonstrates that eNOS protein expression cannot explain the reduction in NO bioavailability with advancing age [19, 53–55]. Rather, it appears that alterations in eNOS activation status, substrate/cofactor availability and/or inactivation of NO underlie the age-related reduction in NO bioavailability and impairments in EDD. Recent evidence also suggests that prostanoid vasodilators, such as prostacyclin, are diminished with advanced age [56, 57] and may explain some of the reductions in vasodilation. Furthermore, it is appreciated that reductions in NO promote enhanced vasoconstriction mediated by endothelial derived ET-1 [58, 59]. It has been shown that aging results in elevated expression of ET-1 and cyclooxgenase (producer of vasoconstrictor prostaglandins) [19, 58, 60, 61] and this elevation suppresses endothelial vasodilation in older adults and rodent models [19, 62]. Next we will delineate the two macro mechanistic processes which directly lead to suppression of endothelial function in older adults.

3b. Aging, Oxidative Stress and Endothelial Dysfunction

In humans, sedentary aging is considered a state of chronic, systemic "oxidative stress" [63] based primarily on observations of age-related elevations of plasma oxidative stress markers [21, 64, 65]. A key mechanism underlying age-associated reductions in EDD and NO bioavailability is the development of vascular oxidative stress [21, 49, 66–69]. Oxidative stress can be defined as a state in which the bioavailability of reactive oxygen species (ROS) is increased relative to antioxidant defenses [70–72]. Superoxide anion (O_2^-) is a ROS produced by oxidant enzyme systems (NADPH oxidase-p67 phox, xanthine oxidase, cytochrome P450 2C9, uncoupled eNOS) [70, 71, 73, 74] that can inactivate NO [71, 75]. Interestingly, O_2^- can also be produced in abundance in the mitochondria at complex 1 and

3 [76]. However, mitochondrial O_2^- is released in the mitochondrial matrix (complex 1) or the intermembrane space (complex 3). Due to its highly reactive state, it is unlikely to navigate through multiple membranes to the cytosol in abundant quantities without being transformed to a less reactive form of ROS, such as hydrogen peroxide (H_2O_2). Nevertheless, O_2^- in the cytosol or extracellular space interacts with NO to produce peroxynitrite ($ONOO^-$), a ROS that itself nitrates cellular proteins forming nitrotyrosine, which can be used as a robust marker of cellular oxidative stress and $ONOO^-$ production [77–79]. Aging is associated with an increased abundance of arterial nitrotyrosine in animal models [29, 55, 80] and in arterial endothelial cells of humans [22]. In addition to the nitration of tyrosine which is irreversible, aged arteries also exhibit markers of lipid peroxidation such as 4-hydroxynonenal (4-HNE) or malodialdehyde (MDA) and/or other reversible regulatory oxidative stress marker glutathionylation at cysteine residues [81–84]. Collectively, these oxidative stress-driven biochemical events result in a reduction in NO bioavailability and, subsequently, impaired EDD [66, 68].

Evidence of oxidative stress-associated suppression of NO and EDD in older arteries is supplied by the observations that acute administration of antioxidants, such as vitamin C and superoxide dismutase (SOD) mimetics, that reduce O_2^-, nearly unilaterally improve NO bioavailability and EDD in older animals and humans [21, 66, 85]. An important source of increased vascular O_2^- and oxidative stress with aging appears to be NADPH oxidase [22, 55, 75]. Animal studies have shown similar improvement in EDD after blockade of NADPH oxidase as that seen with the use of SOD mimetics [55, 86]. Uncoupled eNOS is another important source of O_2^-. eNOS uncoupling occurs when the critical cofactor BH4is inadequate, leading eNOS to produce O_2^- instead of NO [87]. Reducing uncoupled eNOS, by restoring BH_4, improves EDD in older adults by reducing oxidative stress-mediated suppression of EDD [88]. This has been further supported by the observation of reduced O_2^- after BH_4 administration in *ex vivo* arterioles from old rats [89]. In contrast, we have not found an aging-related increase in expression and/or activity of the other major oxidant enzymes (i.e., xanthine oxidase and cytochrome P450) in the aorta of mice or in vascular endothelial cells obtained from humans [22, 55, 90, 91]. Furthermore, studies using pharmacological inhibitors of xanthine oxidase and cytochrome P450 2C9 have resulted in no improvement in EDD in older adults [90, 92]. Interestingly, mitochondrial ROS has been shown to contribute to the ROS production/spillover in arteries from old rodents. However, this mitochondrial ROS is in the form of H_2O_2 and not directly as O_2^- [93] and is likely due to the production of O_2^- in the mitochondrial matrix. Still, targeting mitochondrial O_2^- reduces arterial O_2^-, increases NO and improves EDD [94, 95]. This can be done either through genetic deletion of the mammalian Shc adaptor protein, $p66^{shc}$ (a protein that can increase mitochondrial H_2O_2 production and inhibit FoxO3a activity) or by introducing exogenous antioxidants targeted to the mitochondria, e.g. MitoQ.. Likewise, old MnSOD haploinsufficient mice have impaired EDD compared with old wildtype mice [96], supporting the importance of mitochondrial O_2^- in aged arteries. The mechanism of action of this phenomenon has yet to be elucidated, but does suggest that O_2^- produced in the mitochondria in excess indirectly alters NO, an effect that may result from the influence of H_2O_2 on cytosolic or extracellular O_2^- producers, such as NADPH oxidase [97] or from

cycling of H_2O_2 into other ROS, such as Fenton reaction production of OH^- that then maybe able to inactivate NO.

The contribution of reduced antioxidant defenses against the development of systemic and vascular oxidative stress with sedentary aging is less clear. Circulating markers of antioxidants have been shown to be either reduced [64, 65] or unchanged with aging in humans [21]. Similarly, reduced expression of antioxidant enzymes, including intracellular and extracellular isoforms of SOD, have been observed in the vasculature of old animals [98, 99], although not in all cases [100, 101]. Furthermore, the concentration of glutathione peroxidase, a critical regulator of H_2O_2, has been shown to be similar between young and old arteries [96, 102] though mice which lack glutathione peroxidase have potentiated arterial dysfunction (both smooth muscle and endothelial) and oxidative stress in late adulthood (1 yr. old) [81]. Our human endothelial cell data suggests similar levels of SOD (CnZn & Mn) and catalase between populations of healthy older adults and young controls [22]. However, it has been reported that nitration of MnSOD may independently reduce its activity [103] and nitration of MnSOD is elevated in middle-aged and old rat aortas [53], unfortunately activity of Mn SOD was not measured. Still, in two independent studies we have demonstrated similar MnSOD activity from young and old mice in whole aortic homogenates [29, 104]. Therefore, it remains unclear if this nitration of Mn SOD has functional consequences in aged arteries or if Mn SOD activity is reduced in older endothelial cells. A summary age-related endothelial cell changes is provided in Figure 1. Taken together, these findings indicate that the expected increase in the expression of antioxidant enzymes that would be appropriate in response to increased ROS production with aging does not occur and thus an inappropriate transcriptional response to enhance antioxidant defenses likely contributes subsequent oxidative stress.

One caveat to the interpretation of the age-related finding above is that this excess O_2^- produced with aging is deleterious, whereas a normal amount of O_2^- production is essential for transcriptional signaling. Furthermore, O_2^- is actually essential for intact EDD in resistance arteries from young rodents and older rodents that have undergone exercise training or caloric restriction, as indicated by impaired EDD in response to SOD mimetics [86, 102, 105]. Likewise, antioxidants reduce brachial artery dilation to handgrip exercise in exercise-trained older adults [106]. Furthermore, some ROS, such as H_2O_2, are potent vasodilators that can compensate for reductions in NO [107, 108]. Thus, it is clear that ROS are necessary for proper arterial function, but when out of balance, can induce a critical dysfunction in the vascular endothelium.

3c. Aging, Inflammation and Endothelial Dysfunction

Initial observations made in circulating plasma suggested that aging was associated with chronic, low-grade inflammation characterized by increases in circulating acute phase proteins (e.g., C-reactive protein [CRP]) and pro-inflammatory cytokines [22, 91, 109], such as tumor necrosis factor alpha (TNF-α) [110] and interleukin (IL)-6 [22, 91, 111]. More recently, it has been demonstrated that expression of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and interferon gamma (IFN-γ) are also elevated in the large elastic arteries of old mice [112, 113] and humans [114]. This age-associated pro-inflammatory arterial

phenotype is downstream of increased nuclear factor κB (NFκB) activity. NFκB is a transcription factor that resides in the cytoplasm through its interaction with the inhibitory protein, I kappa B-alpha (IκB-α)[115, 116]. In response to inflammatory stimuli [116] or ROS [117–119], I kappa B kinase beta (IKKβ) is activated and subsequently phosphorylates IκB-α, releasing its inhibition and allowing NFκB to translocate into the nucleus where it can activate gene transcription of pro-inflammatory cytokines [120]. Supporting the critical role of NFκB in age-related inflammation dependent endothelial dysfunction is evidence that pharmacological inhibition of NFκB signaling significantly reduces cytokines and enhances EDD in old mice and humans [113, 121, 122].

This pro-inflammatory arterial phenotype has been demonstrated in the vascular endothelial cells of older healthy humans [22, 91] and underlies endothelial dysfunction in older humans and mice [113, 121]. Evidence for a direct role of inflammation in endothelial dysfunction is provided by studies in which exogenous administration of pro-inflammatory cytokines was shown to induce endothelial dysfunction or endothelial activation in primary endothelial cells or isolated arteries [123–125]. Such an inflammation-mediated endothelial dysfunction has also been observed *in vivo* in carotid arteries of aged rats [52] and in adipose tissue arteries of mice with diet-induced obesity [125], with the dysfunction occurring downstream of elevated TNF-α. *In vivo* arterial TNF-α may be produced locally by vascular cells [52, 112] or by immune cells infiltrating the adventitia of the large arteries [112]. TNF-α associated pro-inflammatory signaling may occur both up- and down-stream of elevated NFκB activity which itself underlies vascular dysfunction, at least in part, via increases in oxidative stress [52, 112, 113].

In addition to exacerbating inflammation downstream of NFκB transcription of pro-inflammatory cytokines, inflammatory signaling also stimulates O_2^- production and oxidative stress (and vice versa) through a number of mechanisms. These include increased NFκB mediated transcription of redox-sensitive genes like those encoding subunits of NADPH oxidase [126–128] that increase ROS bioactivity and further activation of IKK-NFκB signaling. Thus, NFκB lies at the center of a vicious cycle that can exacerbate oxidative stress and inflammation (Figure 2). Interestingly, endothelial NFκB can impact the healthspan/lifespan beyond its effects on vascular function *per se*. Indeed, inhibition of endothelial NFκB signaling protects against not only age-associated vascular senescence and oxidative stress, but also protects against diet- and age-associated insulin resistance and increases lifespan in a mouse model expressing a dominant negative IKK in the endothelium [129].

In addition to NFκB signaling, there are a number of other inflammation-sensitive pathways that may also be involved in age-associated vascular dysfunction. Inflammatory and growth factor stimuli destabilize the vascular endothelium [130–132] via activation of the small GTPase, ADP-ribosylation factor 6 (ARF6), its activator ARF nucleotide binding site opener (ARNO) and the downstream GTPase, Rac [132]. ARF6-ARNO-Rac act to reduce endothelial cell-cell interactions promoting vascular permeability, leukocyte adhesion and angiogenesis [132–134]. Dysregulation of this inflammation-sensitive pathway can lead to disruption of normal endothelial barrier function, vascular leak, tissue disruption and pathological angiogenesis in retinal vascular disease [132, 134]. However, the impact of

aging on this pathway and its role in vascular aging are unknown. Similarly, inflammatory signaling activates the Notch pathway to inhibit cell growth and promote hyper permeability and cell senescence (the implications of which will be discussed later in this review)[135, 136]. Interestingly, Notch signaling is enhanced in atherosclerotic regions of aortas from mice and humans and is activated in endothelial cells of older adults [135]. Notch activity is also increased in models of accelerated aging in response to progerin expression, a mutant "prelamin A" protein [137, 138]. Moreover, expression of the progerin protein is elevated in vascular tissues and cells from otherwise healthy aged humans [139, 140], suggesting that augmented Notch signaling may underlie at least some of the effects of vascular aging [141]. Thus, in addition to NFκB, other inflammation-sensitive pathways also have the potential to play a role in vascular dysfunction, e.g. impaired endothelial barrier function/vascular hyperpermeability [26, 31, 142, 143] and increases in cell senescence, all of which are associated with advancing age.

4. Cellular Senescence and Genomic Instability

4a. Cellular Senescence in Endothelial Aging

Cellular senescence, or permanent cell cycle arrest, is considered cellular aging, as it occurs *in vitro* after a certain number of cell cycles and in response to excessive intracellular and extracellular stressors [144, 145]. Senescence primarily occurs in the G0/G1 phase of the cell cycle and is a vital tumor suppressive mechanism that prevents passing damaged DNA to daughter cells or potential neoplastic transformation of damaged cells [144, 145]. Since being first described by Leonard Hayflick as an *in vitro* phenomenon in human fibroblasts, the potential role of senescence in *in vivo* aging and disease has been difficult to assess and somewhat controversial [146]. However, recent studies have shown that senescent cells accumulate in normal arterial tissue over the lifespan of humans [147, 148]. Likewise, the accumulation of senescent cells has been reported in diseased tissues, such as atherosclerotic plaques [149] and abdominal aortic aneurysms [150]. Baker *et al.* showed that clearance of senescent cells reversed aged and diseased phenotypes in a mouse model of accelerated aging [151]. This important study strongly suggested that there were phenotypic properties of senescent cells that were problematic to tissues, and potentially contribute to aging and chronic disease.

There are several causes of cellular senescence in mammalian cells, including excessive mitogenic signals [152], increases in extracellular or intracellular stressors like oxidative stress[153], chromatin disruptions [154], and DNA damage [155]. Senescence is induced by the two major tumor suppressor pathways, known as the p53 and p16/pRB pathways [156]. The p53 pathway depends on activation of the transcription factor p53 by a number of different signaling cascades [156]. One of the most important of these is the DNA damage response pathway [155, 157]. The p16/pRB pathway involves cyclin-dependent kinase inhibitors 2A (p16)-mediated inhibition of retinoblastoma-like protein 1 (pRB)[158]. The preference toward one pathway versus another appears to be cell type-specific [156, 159], with variation across species [160]. For example, telomere dysfunction can lead to activation of the p53 or p16/pRB pathway in human cells, but will only trigger the p53 pathway in rodent cells [160]. The general consensus seems to be that senescence via the p53 pathway

is activated primarily by DNA damage and telomere dysfunction, while the p16/pRB pathway is linked primarily to mitogenic stress, chromatin disruptions, or general cellular stress [156, 159, 161].

In vitro senescent cells are characterized by a pro-inflammatory, pro-oxidative senescence-associated phenotype (SASP)[144]. The release of inflammatory mediators and ROS production likely reinforces cell cycle arrest in an autocrine fashion and activates immune cell surveillance of senescent cells [144, 162]. The SASP occurs within a few days of senescence induction in cells and appears to be irreversible due to stable chromatin modifications around clusters of SASP genes [163–165]. The SASP profile in vascular endothelial cells from humans and rodents has been characterized by *in vitro* studies using comprehensive arrays of inflammatory cytokines and chemokines [144, 165–169]. The release of IL-6, IL-1, IL-8, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) has been linked to p53-mediated senescence in human vascular cells *in vitro* [170, 171]. Interestingly, P16/pRB-induced senescence has not been shown to lead to a SASP in human cells [144, 164]. Additionally, human arterial endothelial cells that had undergone replicative senescence *in vitro* exhibited elevated levels of H_2O_2 and O_2^- as well as reductions in NO [172, 173]. This SASP profile is possibly due to mitochondrial uncoupling or alterations in eNOS [172, 174]. Several lines of evidence support a major role for NFκB in the induction and maintenance of the p53-mediated SASP in human and rodent cells [175–177]. Indeed, inhibition of NFκB allowed cells to escape p53-mediated senescence and reduced oxidative stress in rodents [175, 177]. This alludes to the importance of the SASP in reinforcing p53-mediated senescence and the importance of NFκB in promoting the SASP. Additionally, p53 activation leads to a shift from glycolytic to oxidative phosphorylation energy metabolism [178], which could result in increased flux through the electron transport chain and ROS release from mitochondria. Taken together endothelial cell senescence could be cause or consequence of the age-related increase in oxidative stress and likely contributes to the spread of vascular inflammation via the SASP, from senescent cells (Figure 3). Lastly this chronic inflammation could persist and accumulate, indefinitely in the vasculature until removal of the senescent cells, thus limiting therapeutic options.

4b. Aging, Genomic Instability, Cellular Senescence and Endothelial Dysfunction

Genomic instability, or DNA damage acquired over time, is an important mechanism that may underlie the age-related accumulation of senescent cells reported in arterial tissues [147, 148]. Age-related genomic instability in vascular cells can occur from a variety of genotoxic insults, including oxidative stress [179, 180] and mechanical stress [181]. DNA damage results in temporary cell cycle arrest to allow DNA repair pathways time to repair breaks prior to a cell entering S phase during replication [155]. If damage is persistent or extensive, permanent cell cycle arrest or even apoptosis will ensue [155]. Common forms of DNA damage relevant to aging include single and double strand DNA breaks and DNA adducts. While the age-related accumulation of DNA breaks and DNA adducts have not been shown in human vascular cells, DNA breaks have been shown to occur with advancing age in sperm cells [182] and the mitochondrial DNA of skeletal muscle [183, 184]. Microsatellite instability and loss of heterozygosity, which is thought to be a consequence of DNA breaks, has been linked to pulmonary artery hypertension [185] and atherosclerosis

[186–191] in humans. Interestingly, two recently developed mouse models of disrupted DNA adduct repair (*xeroderma pigmentosum D (XPD)*^{ttd} and *ERCC1*$^{d/-}$ mice) demonstrated age-related impairments in EDD in response to acetylcholine, reduced eNOS expression and activity, and increased p53 expression and ROS content [192].

Telomere dysfunction is another form of genomic instability that may lead to cellular senescence with advancing age. First described by Harley *et al.*, senescence triggered by replication-dependent telomere dysfunction is often referred to as replicative senescence [193]. Telomeres can be damaged by the genotoxic stressors described above [194, 195], and *in vitro* studies in various human cell types have clearly shown that the breakdown of telomere structure, referred to as telomere uncapping, leads to p53 activation and senescence [196, 197]. The only previous study to measure arterial telomere uncapping reported greater uncapping with advancing age, which was linked to p53-mediated senescence [148].

A vicious cycle of oxidative stress leading to DNA damage or telomere uncapping-mediated p53 activation and subsequent senescence-associated oxidative stress is a simple model of the role of genomic instability in age-related endothelial dysfunction. However, a more integrative model might consider the influence of p53-mediated shifts in energy metabolism on the following [178] energy sensitive pathways: SIRT-1, AMPK and mTOR that are known to influence both aging and endothelial function and that are discussed next.

5. Energy Sensing Longevity Pathways

5a. SIRT-1, Aging and Endothelial Dysfunction

Sirtuins were originally discovered in a screen for gene silencing factors in yeast and, therefore, given the name Sir2 (silent information regulator 2). Little research was conducted on the Sir2 family until Guarente *et al.* identified these proteins as critical regulators of longevity [198–200]. Thereafter, Sir2 and its mammalian homologues, the sirtuin family (SIRT1-7), of NAD+-dependent protein deacetylases and ADP-ribosyltransferases were quickly identified. In mammals, SIRT1-4 have been implicated in the control of cellular metabolism with SIRT-2, 3 and 4 predominantly expressed in the mitochondria and SIRT-1 expressed in the nucleus, with some cytoplasmic expression [201–203]. The sirtuin family and specifically SIRT-1 are implicated in a majority of the physiological benefits of caloric restriction [203–205]. SIRT-1 expression decreases in a multitude of tissues with advancing age [206–208]. SIRT-1 function is related to deacetylation and thereby modulating activity of nuclear transcription factors, co-regulators and proteins to adapt gene expression in response to the cellular energy state and provide “stress resistance” by reducing pro-inflammatory and oxidative stress pathways [209]. Interestingly, activation of SIRT-1 with the small molecule SRT1720 increases lifespan and preserves glucose tolerance in rodents [210] Taken together these data suggest that SIRT-1 activation may have significant promise for improving endothelial function with aging or CVD.

SIRT-1 has been reported to modulate NO and endothelial function in small and large arteries directly via deacetylation and subsequent activation of eNOS [207, 211]. Our group has demonstrated that SIRT-1 expression and activity decrease with age in the vasculature in

both mice and humans [104, 207, 212, 213], and this is associated with age-related endothelial dysfunction in mice [104, 207]. Interestingly in rodent models, both short and long term caloric restriction can prevent the decline in arterial SIRT-1 and endothelial function [104, 212, 214]. Most of the data related to the vascular endothelium and SIRT-1 activators is restricted to studies utilizing resveratrol, a naturally occurring polyphenol [215]. Unfortunately, resveratrol must be used at high doses to activate SIRT-1 [216] and is known to activate over 15 unique pathways in addition to being a potent antioxidant and phytoestrogen [215, 217–221]. While these characteristics of resveratrol do not alter its potential therapeutic value, they do generate some difficulty in elucidating its mechanism of action, specifically whether SIRT-1 activation is responsible for its beneficial effects. It has been shown that very high doses of resveratrol (2400 mg/kg of food) improves EDD in isolated arteries from middle-aged animals after 1 year of treatment [222], although it is unknown by what mechanism(s) this effect was achieved. Subsequently, we have utilized SRT-1720 treatment in old mice and have demonstrated that four weeks of SRT-1720 normalizes SIRT-1 activity and reduces age-related NFκB acetylation, arterial inflammation and oxidative stress [213, 223]. Somewhat surprisingly SRT-1720 improvements in EDD were not mediated by increased NO bioavailabilty in old mice, but rather via augmented COX-2 vasodilators [213, 223]. Therefore, despite strong evidence suggesting a role of reduced SIRT-1 in the age-related reduction in NO bioavailability and endothelial dysfunction, treatment with a SIRT-1 activator improves endothelial function and reduced oxidative stress and inflammation, but does not restore NO. Future studies will be needed using small molecule activators of sirtuins to determine if they can ameliorate age-related CVD pathologies.

5b. AMP-activated protein kinase (AMPK), Aging and Endothelial Dysfunction

AMPK, a highly conserved heterotrimeric serine-threonine kinase, is an important energy sensing signaling protein that integrates energy balance, metabolism and stress resistance [224, 225]. AMPK is activated in response to increases in the AMP:ATP ratio and following physiological stimuli such as shear stress, heat shock, exercise and hypoxia [226]. AMPK is made up of a catalytic α subunit, a structural β subunit and the AMPK binding site containing γ subunit, each of which exist in multiple isoforms [226]. Activation of AMPK requires phosphorylation of the α subunit and occurs downstream of two kinases; LKB1 and Ca2+/calmodulin-dependent protein kinase kinase β (CaMKKβ) (reviewed elsewhere [226, 227]). Shear stress-induced AMPK activation in endothelial cells is independent of Akt and involves the activation of the α_2 subunit via LKB1 [226]. AMPK activation can also be mediated pharmacologically with aminoimidazole carboxamide ribonucleotide (AICAR) or metformin. AICAR is a direct activator of AMPK that acts downstream of LKB1 leading to phosphorylation of the α2 subunit of AMPK [226]. In contrast, metformin is an indirect activator of AMPK [228], actions of which are mediated by an increase in the AMP:ATP ratio resulting from inhibition of Complex I of the respiratory chain [229, 230]. However, such inhibition of oxidative phosphorylation, and a subsequent increase in glycolysis, can have numerous effects independent of AMPK, e.g., metformin effects transcription factors and kinases involved in numerous cell cycle and metabolic pathways (p53, p38, MAPK, PKC and Akt)[231–234]. Therefore, as with resveratrol, metformin and other non-specific

agents are not optimal for mechanistic studies evaluating these pathways but do afford insights as potential therapeutic agents.

AMPK activation in vascular endothelial cells can contribute to both angiogenesis and NO production. Angiogenic effects of AMPK occur both upstream and downstream of vascular endothelial growth factor (VEGF), with the effects of AMPK signaling promoting differentiation of endothelial progenitor cells [235] as well as angiogenesis in isolated myocardial microvascular endothelial cells [236], in ischemic skeletal muscle [237] and during hypoxia both *in vitro* and *in vivo* [238]. AMPK activation also results in increased activation of eNOS [239, 240] via signaling through Rac1 and Akt [238, 240], as well as through direct phosphorylation of eNOS [241, 242]. While the pro-angiogenic effects of AMPK have been linked to increased VEGF expression [236, 237], AMPK has also been demonstrated to transcriptionally regulate a number of other proteins involved in inflammation, mitochondrial biogenesis, fatty acid and cholesterol synthesis, glucose metabolism, cell growth and oxidative stress signaling [226], all of which may impact vascular function. Taken together, these findings suggest that augmenting AMPK signaling may not only enhance angiogenesis and increase bioavailability of NO and vasodilatory responses [235, 238, 240], but may also impact upstream mediators of age-associated endothelial dysfunction, such as inflammation and oxidative stress. Indeed, AMPK activation improves endothelial function in type I and II diabetic rodents [243–245] but, less is known about the activation state of AMPK in aged arteries or its role in age-associated vascular dysfunction.

In the context of vascular aging, although there is a report of increased AMPK activation in endothelial cells cultured to senescence, a cellular model of aging [246], AMPK activity is reduced in the aorta[247] and cerebral arteries [248] of old rodents. Furthermore, the pharmacological activation of AMPK by AICAR increases EDD in old mice [247], suggesting that inactivation of arterial AMPK contributes to age-associated endothelial dysfunction. However, despite evidence for AMPK-mediated eNOS activation [238, 240–242], the effect of *in vivo* AMPK activation by AICAR to improve dilation in arteries from old mice was not mediated by an increased NO bioavailability [247]. Similar NO independent effects of AICAR were also found after acute *in vitro* administration to isolated aortic rings, in which AICAR induced relaxation of agonist constricted vessels independent of NO [249]. Similar to treatment of old mice with SIRT-1 activators, AMPK activators therefore reduce arterial oxidative stress and improve endothelial function, but do so in an NO independent mechanism.

5c. Mammalian target of rapamycin (mTOR), Aging and Endothelial Dysfunction

Rapamycin was discovered more than 30 years ago from an Easter Island soil sample. It is a potent antifungal metabolite produced from bacteria and in addition to its antifungal properties was quickly found to have antiproliferative and immunosuppressant properties when used in high quantities. The mammalian target of rapamycin (mTOR) is a signaling protein which responds to nutrients (i.e. amino acids) or growth factors (i.e. insulin) in order to modulate mRNA translation, protein synthesis and cellular growth [250]. mTOR signaling is composed of two distinct pathways the mTOR complex 1 (mTORC1), the more

typically studied and rapamycin “sensitive”, and mTOR complex 2 (mTORC2), the less clearly identified and less sensitive to rapamycin. Most information to date on the role of mTOR has studied the insulin/nutrient signaling via the mTORC1 and significantly less in known about the role of mTORC2 (in this review, future references measure either mTORC1 or general mTOR activity)[251]. Earlier this decade studies showed that decreasing TOR signaling, genetically or with rapamycin, in yeast, *Drosophila*, and *C. elegans* is able to slow aging and increase lifespan [252–255]. Follow-up studies out of Richard Miller’s laboratory reproduced these findings in mice fed a diet with rapamycin incorporated [256, 257]. These studies suggested that inhibiting mTOR via rapamycin could delay age-associated diseases and extend lifespan in mammals. A subsequent study replicated these findings by genetically manipulating a downstream target of mTOR, ribosomal S6 protein kinase (S6K1) and demonstrated a similar phenotype of reduced risk factors for age-related diseases (bone density, insulin sensitivity) and increase lifespan [258]. These studies unilaterally implicate elevated mTOR signaling in accelerated aging and associated diseases. Currently, very little is known about mTOR and endothelial function. We have demonstrated that mTOR signaling is augmented in arteries from older mice which display endothelial dysfunction and lifelong caloric restriction prevents augmented arterial mTOR signaling and endothelial dysfunction [104]. Furthermore, preliminary studies in our lab suggest that 6–8 weeks of dietary supplementation of rapamycin improve NO and endothelial function in old mice [259]. Chronic rapamycin and other rapamycin analog studies are warranted to determine if inhibition of the mTOR pathway is a viable method to improve endothelial function in older adults.

6. Interactions of mTOR, SIRT1 and AMPK: Role in the Vascular Aging Phenotype

Although the energy sensor pathways, SIRT-1, AMPK activation and mTOR inhibition have been linked to the vascular aging phenotype, when viewed from a wider perspective, what becomes evident is that these pathways do not, in fact, act in parallel to independently impact the macromechanistic processes (e.g. inflammation, oxidative stress and senescence) associated with vascular dysfunction. Rather, there are points of convergence and crosstalk among these pathways that not only impact the downstream effects, but also the expression of the signaling molecules themselves. Indeed, signaling through the transcription factors *NFκB, FoxO and p53, represent points of convergence for signaling downstream of mTOR, SIRT-1 and AMPK.*

6a. NFκB

The acetylation of NFκB p65 subunit by p300 prevents the nuclear export of NFκB, while its deacetylation by SIRT-1 allows for the association of NFκB with IκB-α and its subsequent nuclear export and inactivation [260]. Inhibition of mTOR (complex 1) by rapamycin inhibits phosphorylation of NFκB and subsequent nuclear translocation via its influence on IKKβ activity [261–263]. AMPK signaling has a complex interaction with NFκB [264], such that AMPK can both indirectly inhibit pro-inflammatory NFκB signaling [265–267] and activate NFκB signaling leading to its anti-apoptotic effects in endothelial cells [268]. Taken together these studies indicate that SIRT-1, mTOR and AMPK can all

alter the pro-inflammatory transcription factor NFκB activity by both distinct and intersecting pathways. It also indicates that reductions in AMPK and SIRT-1 activity and conversely increased mTOR activity may increase NFκB signaling resulting in a pro-inflammatory phenotype similar to that observed in older arteries

6b. FoxO transcription factors

Fox nuclear transcription factors are a family of proteins characterized by a conserved 100 amino-acid sequence known as the “forkhead box.” The FoxO subgroup have been implicated in ROS detoxification, cell cycle regulation, apoptosis, and DNA repair [269]. The activity of this subgroup is dependent on posttranslational modifications, such as phosphorylation and acetylation. FoxO proteins are constitutively held in the cytoplasm, and phosphorylation by signaling kinases inhibit their transcriptional activity by preventing nuclear translocation [270]. Phosphorylated FoxO is retained in the cytoplasm by the 14-3-3 chaperone protein, and phosphorylation at serine residues 256/253 [271] targets FoxO proteins for degradation via ubiquitination [272]. The downstream targets of nuclear FoxO activity are determined by acetylation [273]. For example, p300-mediated FoxO3a acetylation induces the preferential transcription of pro-apoptotic gene targets. SIRT-1 deacetylates FoxO3a, leading to the preferential transcription of genes related to stress resistance, such as the antioxidants MnSOD and catalase [273–275]. Furthermore, the FoxO4 isoform is an endogenous NFκB inhibitor, whose activity is enhanced when bound to SIRT-1 [276]. The interaction of mTOR and FoxO signaling occurs through modulation of other signaling kinases, such as protein kinase B (Akt) and serum- and glucocorticoid-regulated kinase-1 (SGK1). The phosphorylation of FoxO proteins by these kinases leads to the cytoplasmic retention and inactivation of FoxO transcription factors [277]. Rapamycin, an inhibitior of mTOR, can inhibit SGK1 activity downstream of the mTORC1 complex [278] and may, as a result, increase FoxO-mediated transcription of anti-oxidant gene targets. AMPK can also directly phosphorylate the 3a isoform of FoxO leading to its activation and subsequent inhibition of NFκB [279].

6c. p53

p53 is a transcription factor that acts as a tumor suppressor preventing the survival of malignant cells. p53 is activated by stress such as DNA damage, telomere attrition and hypoxia and leads to cell cycle arrest and apoptosis [280]. Endothelial dysfunction in older adults is associated with telomere dysfunction and subsequent increases in p53-mediated cell cycle arrest via increases in the cyclin dependent kinase inhibitor, p21 expression [148]. Furthermore, recent evidence points to a link between the DNA damage response and energy sensitive pathways via SIRT-1 activity and expression. SIRT-1 is involved in the DNA repair and its activity has been shown to protect human and rodent vascular cells from DNA damage in the context of atherosclerosis [281]. Thus, age-related reductions in endothelial SIRT-1 expression and activity [207] may be partly the result of depleted protein levels caused by increased need for repair of accumulating DNA damage. Conversely, reductions in endothelial SIRT-1 expression may lead to genomic instability with advancing age. Specifically SIRT-1 deacetylates p53 resulting in its inactivation and the promotion of cell survival, thus reductions in SIRT-1 activity with aging may contribute to p53 activation and increased cell senescence [282, 283]. Conversely, AMPK phosphorylates p53 on ser15

and ser20 [284, 285] leading to p53 stabilization, activation and cell cycle arrest [286]. Phosphorylation of p53 at these sites has also been associated with reductions in inflammation [284]. Although, AMPK induced decreases in cell senescence does not likely contribute to the aging vascular phenotype, a loss of the p53-associated anti-inflammatory effects of AMPK may be an important factor. The crosstalk between p53 and mTOR occurs in the inverse manner as described for SIRT-1 and AMPK, with p53 inhibiting mTOR activity [287]. However, as both p53 and mTOR activation is increased with vascular aging, it is unlikely that this inhibitory interaction is a significant contributor to the vascular aging phenotype.

7. Conclusions

Since the initial observations of endothelial dysfunction in older adults in the early 1990s, the field has achieved several significant milestones. First, due to initial work and reviews by early researchers, vascular aging now is appreciated as a critical step in the development of age-related CVD. This recognition has allowed for funding and expansion of the field of study. Second, the vascular aging research community has expanded dramatically in the last 20 years and has established viable primate and rodent models that phenocopy human aging. These models have allowed for greater mechanistic insight into the cellular and molecular mechanisms of endothelial dysfunction as well as the translation of interventions to human endothelial physiology studies. Third, in the past 25 years, the research community has identified multiple pathways that directly influence oxidative stress and inflammation, the major mechanisms of endothelial dysfunction with advancing age. Emerging evidence has suggested several promising pathways that could be viable candidates to reverse age-related endothelial dysfunction that require further exploration in areas outside of endothelial dilation; such as fibrinolysis, permeability and angiogenesis. Unfortunately, it is still unknown what the initiating events are that alter the cellular machinery to induce the well described vicious cycle of oxidative stress and inflammation. Indeed, although deregulation of the energy sensing pathways, e.g., SIRT-1, mTOR and AMPK, are attractive possible mechanisms, to date, pharmacological interventions that restore normal pathway activity in older animals have proven only partially effective at restoring endothelial function to that of young animals. Finally, treating older adults with prescription drugs to improve endothelial function as a preventative measure against CVD is not currently acceptable in the absence of clinical disease. Unless the definition of “clinical disease” is extended to include endothelial dysfunction, the only viable preventative interventions are those involving lifestyle changes or treatments with nutraceuticals. Overall, despite impressive advancements in our understanding of the biology of aging in the endothelium, we as a research community still have fundamental questions to answer including: What are the initiating events that lead to the arterial aging phenotype? And if a target pathway can be identified and viable drugs created to improve endothelial dysfunction in older adults, when should such therapy be initiated?

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Research Highlights

- Aging and endothelial dysfunction are risk factors for cardiovascular disease
- Both oxidative stress and inflammation suppress endothelial function in older adults
- Genomic instability and senescence are present in older arteries
- Dysregulated energy sensing pathways contribute to the age-related endothelial phenotype
- The initiating events leading to age-related endothelial dysfunction are still unknown

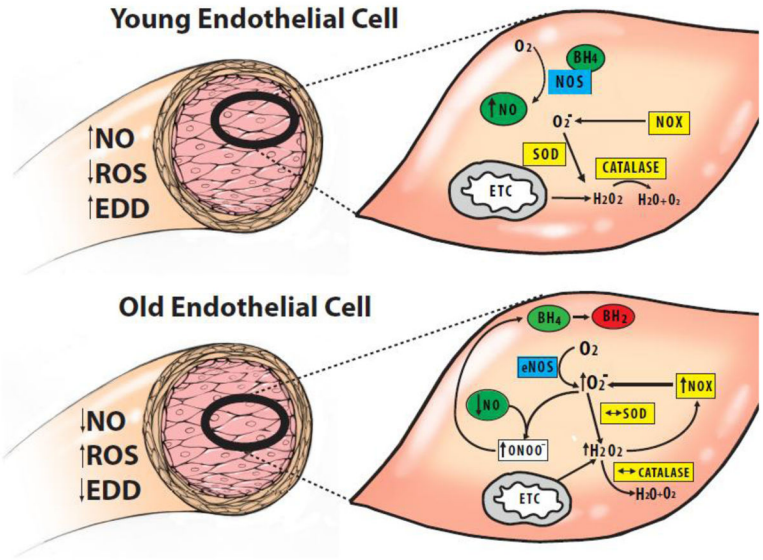

Figure 1. Age-associated Endothelial Oxidative Stress and Impaired NO Bioavailability

In younger endothelial cells (**Upper Panel**), endothelial nitric oxide synthase (eNOS) has adequate cofactor availability, e.g., tetrahydrobiopterin (BH_4), and produces nitric oxide (NO) through the conversion of L-arginine to L-citrulline. Reactive oxygen species (ROS), e.g., superoxide (O_2^-) and hydrogen peroxide (H_2O_2), produced by the mitochondrial electron transport chain (ETC) or cytosolic oxidant enzymes, such as NADPH oxidase (NOX), are quenched by endogenous antioxidant enzymes (superoxide dismutase [SOD] and catalase). In older endothelial cells (**Lower Panel**), ROS produced in the mitochondria increase NOX mediated O_2^-, this quenches NO bioavailability, through its conversion to peroxynitrite ($ONOO^-$), as well as uncouple eNOS by reducing BH_4 availability. In the face of unchanged antioxidant defenses, these effects lead to a reduction in NO bioavailability and a pro-oxidant phenotype in the aged endothelium.

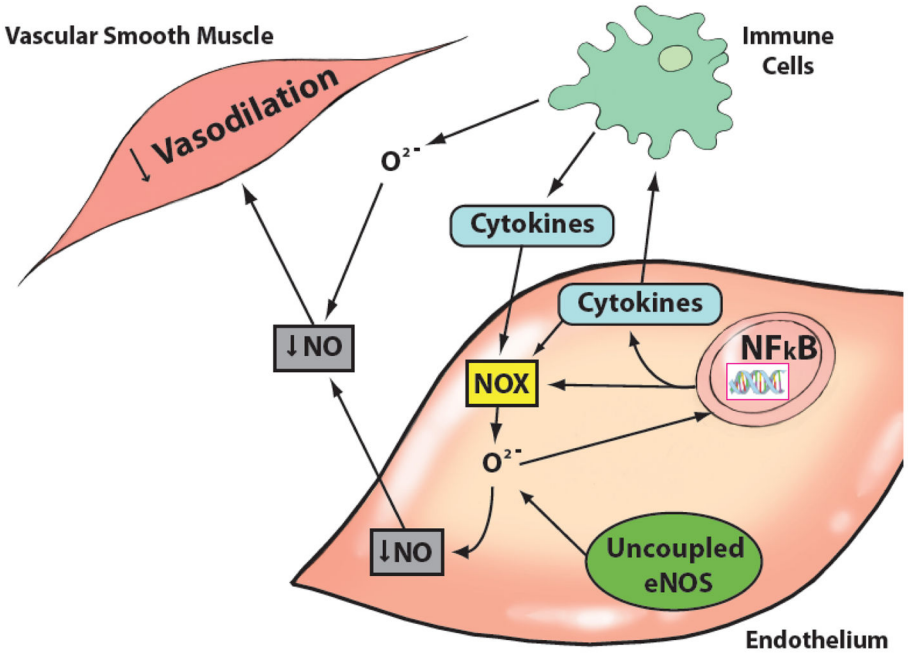

Figure 2. Inflammation and Oxidative Stress in the Aged Endothelium: A Vicious Cycle

The pro-inflammatory transcription factor, nuclear factor kappa B (NFκB) normally resides in the cytosol, where it is inactive, and endothelial nitric oxide synthase (eNOS) produces nitric oxide (NO) that is released from the endothelium and acts on the vascular smooth muscle to cause relaxation. With aging, the endothelial environment is perturbed by increases in cytokines and reactive oxygen species (ROS; e.g., superoxide [O_2^-]), that can both be produced within the endothelium or by neighboring immune cells. These cytokines exacerbate oxidative stress and inflammation in the endothelium by activating oxidant enzymes, such as NADPH oxidase (NOX) increasing O_2^- production, as well as by acting in a feed forward manner to increase pro-inflammatory NFκB transcription. Likewise, the oxidative stress produced in the microenvironment of aged arteries, also acts in a feed forward manner to increase pro-inflammatory NFκB activity and activate neighboring immune cells as well as contributes directly to impaired NO by reducing eNOS activity via decreased BH_4 availability as well as by quenching NO, leading to impaired endothelium dependent dilation in aged arteries. Thus, with aging there is a vicious cycle in aged arteries, in which inflammation and oxidative stress exacerbate one another impairing NO bioavailability and endothelial function.

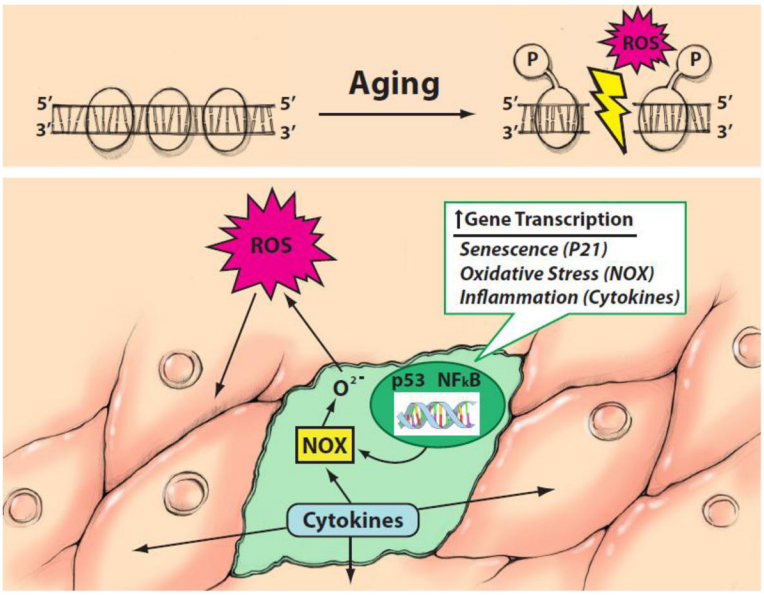

Figure 3. Endothelial Senescence and Aging

With advancing age, reactive oxygen species (ROS), as well as genotoxic stressors and telomere dysfunction, lead to double strand DNA breaks and genomic instability (**Upper Panel**). This genomic instability induces the DNA damage response, leading to the activation, p53 and nuclear factor kappa B (NFκB) that then transcribe genes that contribute to cell senescence such as the cyclin dependent kinase inhibitor, p21; oxidative stress, e.g., NADPH oxidase (NOX); and inflammatory cytokines. The cytokines and ROS act in a paracrine manner to impair function in neighboring endothelial cells (**Lower Panel**).